New applications of alphavirus-based expression vectors

Marco Boorsma^{1,2}, Daniel Koller¹, Wolfgang A. Renner¹ & Martin F. Bachmann^{1*}

¹ Cytos Biotechnology AG, Wagistrasse 21, CH-8952 Zurich-Schlieren, Switzerland

² Institute for Biotechnology, ETH-Zurich, CH-8093, Zurich, Switzerland

(* Author for correspondence; E-mail: bachmann@cytos.com; Fax: +41-1-7334030)

Received 10 January 2001; accepted 1 July 2001

Key words: alphavirus, expression, gene therapy, inducible expression, neurobiology, non-cytopathic replicon, screening, vaccine development

Abstract

Alphaviruses are positive stranded RNA viruses that replicate to extremely high titers. Sindbis and Semliki Forest viral vectors are widely used tools for high-level production of recombinant proteins. Recent studies have broadened their scope to vaccine production, gene therapy, and analysis of cell function. Here we discuss the development of non-cytopathic and inducible expression vectors which can be applied to bioprocess development strategies. Furthermore, a Sindbis-based expression cloning system has been developed that allows for the rapid identification of genes encoding proteins with a selected functional activity.

Introduction

Alphaviruses are positive stranded RNA viruses, belonging to the family of the Togaviruses. The cloning of full-length cDNAs of alphaviruses (Rice et al. 1987; Davis et al. 1989; Liljeström et al. 1991) lead to one of the best-defined animal virus expression systems known. Their biology has been described in various excellent reviews and is therefore only briefly discussed here (Strauss and Strauss, 1994; Schlesinger and Schlesinger, 1996). Cells of both vertebrate and invertebrate origin can get infected via receptor-mediated endocytosis. After RNA release into the cytoplasm, the 5' two-third of the genome encoding four non-structural proteins is translated to form the viral replicase. The replicase amplifies the positive strand genomic RNA via a negative strand intermediate from which high amounts of subgenomic RNA is produced. The subgenomic RNA is represented by the 3' end of the alphavirus genome and encodes the structural proteins, consisting of the capsid and the envelope proteins (Figure 1A). The identification of non-translated *cis*-acting elements sufficient for the RNA replication made it possible to design alphaviral expression vectors that can express virtually any gene of

interest in any replication permissive host cell. In this paper we present the latest applications of alphaviral expression systems.

Alphaviral expression vectors

Several members of the alphavirus group, including Semliki Forest virus (SFV), Venezuelan equine encephalitis (VEE) virus, and Sindbis (SIN) virus, have received considerable attention as expression vectors (Xiong et al. 1989; Liljeström and Garoff 1991; Lundström 1997; Frolov et al., 1996). Because of the cytopathic replication of the virus, the first generation of RNA-based vectors could only be used for transient expression. However, these expression vectors have several advantages. First, the high level replication and host cell protein biosynthesis shut-off lead to high expression levels (Kaariainen and Ranki, 1984). Second, the relatively small size of the positive-stranded alphavirus RNA allows manipulation of the corresponding cDNA by standard recombinant DNA techniques, which is subsequently *in vitro* transcribed into RNA. Third, the replication occurs entirely in the cytoplasm, circumventing RNA export from the nuc-

Figure 1. Structure of the alphavirus and the alphaviral vectors. (A) The alphavirus genome consists of the non-structural proteins (nsp) and the genes for the structural proteins under control of the subgenomic promoter (sp). The 5' non-translated region (NTR) and 3'NTR are recognized by the viral replicase and are required for replication. (B) Alphaviral vectors have been developed by placing a gene of interest (goi) under control of the subgenomic promoter. Viruses can be produced by providing the genes for the structural proteins under control of a second subgenomic promoter in *cis* (double subgenomic), or in *trans* on a defective helper construct (bipartite). Replication competent viral particles are formed when the defective helper RNA contains a packaging signal (ps). Replication deficient particles are produced when the ps is deleted resulting in particles lacking the structural genes.

leus and adventitious splicing (Garoff and Li, 1998), and fourth, their broad host range (Strauss and Strauss, 1994).

Two types of alphaviral expression systems have been described, the double subgenomic and the bipartite system (Figure 1B). In both systems the gene of interest is placed under control of a second subgenomic promoter. The RNA-based vectors are transcribed *in vitro* from a bacteriophage promoter to produce infectious RNA that can be transfected into the cytoplasm by electroporation. The structural proteins can be provided in *cis* by using a second subgenomic promoter on the same construct (Figure 1B, double subgenomic; Raju and Huang, 1991; Hahn et al., 1992), or in *trans* by co-transfection of a second, so

called defective helper construct lacking a functional replicase (Figure 1B, bipartite; Breedenbeek, 1993). Both approaches may result in high viral titers, and the recombinant viruses can infect a new layer of cells leading to high-level expression of the gene of interest. By co-transfecting defective helper constructs lacking the packaging signal, viral particles are produced that do not contain the RNA coding the structural proteins and therefore fail to produce new viral particles.

In the DNA-RNA layered expression systems, a eukaryotic promoter is placed upstream of the alphaviral replicon (Herweijer et al., 1995). After transcription, an RNA molecule is transported from the nucleus to the cytoplasm where it is replicated as during normal infection. Recombinant viral particles

can be produced by co-transfecting a DNA-based defective helper packaging construct, or transfection of packaging cell lines stably expressing the viral structural proteins with the alphaviral replicon containing the gene of interest (Polo et al., 1999).

Vaccine development and anti-tumor strategies

Both RNA- and DNA-based systems are being used and modified for specific applications. A very promising field in which both types of vectors are used is in vaccine production. Important advantages of vaccination with alphaviral particles expressing an antigen are the high expression levels and their broad host range, leading to efficient infection of cells *in vivo*. Moreover, VEE virus preferably infects the professional antigen presenting dendritic cells (MacDonald and Johnston, 2000), resulting in efficient presentation of the antigen to the immune system. The same is achieved for SIN virus vaccines by insertion of a point mutation in the glycoproteins (Gardner et al., 2000). The use of alphaviral particles and naked alphaviral DNA has led to protective immune responses generated against several viruses, including influenza virus (reviewed by Schlesinger and Dubensky, 1999; Fleeton et al., 2001). However, studies focussed on the development of alphaviral-based vaccines against SIV, the simian variant of HIV, have shown some success as well. Monkeys vaccinated with SFV or VEE vectors coding for different SIV antigens produced antigen-specific antibodies and cytotoxic T-lymphocytes (CTL). Upon infection with lethal doses, SIV titers were in some cases reduced to undetectable levels in the pre-immunized animals (Davis et al., 2000; Mossman et al., 1996; Berglund et al., 1997). It will be interesting to see whether protection against primary viral isolates can be achieved.

Various groups have used SIN and SFV-based vectors for anti-tumor therapy. Some examples include the injection of mice with SFV particles expressing variants of the P815 antigen from mastocytoma tumors (Colmenero et al., 1999) or the human papillomaviral proteins E6 and E7, which induce the development of cervical carcinomas (Daemen et al., 2000). The antigens elicited strong CTL responses and mice were protected against tumor challenge. Similar results have been obtained with injection of RNA- and DNA-based SIN and SFV vectors encoding various tumor antigens (Ying et al., 1999; Leitner et al., 2000). Cancer cells injected in pre-immunized mice did not form solid tu-

mors and mice bearing established tumors lived longer after therapeutic immunization than the controls.

An alternative use of SFV for anti-tumor therapy was described by Asselin-Paturel et al. (1999). In this study, established tumors were injected with a SFV vector encoding IL-12. Significant tumor regression was observed, whereas no inhibition of tumor growth was shown when the tumor was injected with a SFV vector encoding a marker protein. It could be concluded that this effect was elicited by anti-tumor activity of IL-12 and not by the apoptosis induced by the alphavirus. Interestingly, another study did show inhibition of tumor growth when injected with SFV-LacZ or SFV-GFP (Murphy et al., 2000), indicating that SFV may also have direct anti-tumor activity. Moreover, *ex vivo* infection of human prostate tissue and prostate cancer cell lines with SFV encoding beta-galactosidase resulted in rapid cell death as well (Hardy et al., 2000).

A third approach in anti-cancer therapy using alphaviruses is indirect immunotherapy. Intraperitoneally growing tumors infected with SFV particles coding granulocyte-macrophage colony-stimulating factor (GM-CSF) resulted in inhibition of tumor growth (Klimp et al., 2001). In contrast to multiple injection with recombinant GM-CSF protein, injection of the viral particles increased numbers of neutrophils and macrophages that were activated to tumor destruction. Although survival of mice was not prolonged, alphaviral-based immunotherapy may have potential in combination with other treatments or by using other immuno-modulating proteins.

The strong replication of the SIN and SFV replicons results in high antigen levels, which are efficiently presented to the immune system. Apoptosis of the alphaviral-infected cells and uptake of these dead cells by professional antigen presenting dendritic cells, plus the formation of double stranded RNA may boost the immune system. Although most protocols still use multiple injections of alphaviral particles to elicit a protective response, numerous studies have suggested alphaviral vectors as efficient alternatives for anti-tumor strategies and the development of vaccines against viruses.

Gene therapy and cell function

Viral vectors are the systems of choice for use in gene therapy applications. Several viruses including retrovirus, adenovirus and Herpes virus have been studied

extensively for transient or stable transduction of different cell lines *in vivo* (Kay et al., 2001). The use of alphaviruses for *in vivo* applications has mainly been focussed on vaccination and anti-tumor studies. However, the high-level and transient mode of expression can also be a safe alternative for short-term correction of mutated genes. Potential gene therapy applications for the brain have been described in which SIN viral particles encoding the beta-galactosidase gene were injected into the brain of mice (Altman-Hamamdžić et al., 1997). Marker gene expression was detected for up to 14 days in neurons. The behavior of the test animals was not changed during this period, suggesting a decrease in cytopathicity of the Sindbis vector in brain cells. Similar experiments were described for SFV particles injected in the brain of rats (Lundström et al., 1999). Expression was detected up to 4 weeks, but the authors ascribe this to the high stability of the beta-galactosidase protein. The RNA could be detected for up to 4 days, confirming the transient nature of SFV vector-based expression.

By targeting viral particles to specific tissues or cell types in the body, the opportunity for gene therapy strategies may be increased. Ohno et al. (1997) introduced the IgG-binding domain of protein A in one of the glycoproteins of SIN viral particles. By the addition of a monoclonal antibody specific for a certain cell type, cells of choice could efficiently be infected. Although this application is being studied for anti-tumor therapies (Lijima et al., 1999), it may also be used for targeting of recombinant particles for the transient correction of mutated genes or the *in vivo* study of gene products.

Alphaviral vectors not only have a potential for gene therapy but they are also important tools for the *in vitro* study of neuron-function as well. Maletic-Savatic et al. (1999) and Ehrengreber et al. (1999) isolated hippocampal slices from rat brains and showed that different neuronal cell types could be efficiently infected and expressed high levels of GFP. These GFP-positive neurons could easily be identified by fluorescence microscopy and examined in morphogenesis studies (Maletic-Savatic et al., 1999). Mutants of SFV were constructed with decreased cytopathicity in hippocampal neurons resulting in increased and prolonged expression of GFP. Together with the new temperature-sensitive, non-cytopathic SFV vectors showing tight regulation of marker protein, these vectors will have interesting applications in neurobiology for physiological analysis of selected gene products (Lundström et al. 2001).

Adult cardiomyocytes are difficult to transfect prohibiting *in vitro* studies. Datwyler et al. (1999) described efficient transduction of isolated adult cardiomyocytes by SIN vectors and found a decreased cytopathic effect in these cells. SIN vectors that are non-cytopathic for heart cells will be important tools for cardiovascular studies, and may even have potential for gene therapy.

Functional genomics

With the completion of the sequencing of the human genome, a great number of new genes have been identified. However, for the majority of genes, the function has not been elucidated. Functional expression cloning is based on the expression of a pool of genes from a library and screening for specific protein function. Thus, expression cloning starts with a pre-defined biological function and ends with the gene sequence, in opposite to the genome sequencing approach.

All expression-cloning methods are based on a similar principle (Okayama and Berg, 1983). The total genetic information of transcribed genes in a cell type or tissue is represented by the mRNA pool. These pools are transformed into a cDNA library by reverse transcription. The cloning of this library into a suitable expression vector allows the expression of all cDNAs by introducing this library into a host cell line. Those cells expressing a desired biological function can be selected, and the cDNA sequence leading to the specific function can be obtained by PCR.

Expression cloning technologies for the screening of human genes have preferentially been developed using eukaryotic expression systems, taking advantage of the correct ribosome binding site, protein folding, topology, and in particular glycosylation. Screening eukaryotic cDNAs was first described more than a decade ago based on the transient expression of cDNA libraries in COS cells using SV40 based expression vectors (Okayama and Berg, 1983; Wong et al., 1985). Although the COS expression system was successfully applied to identify a variety of proteins (Vassar et al., 1999; Simonsen and Lodish, 1994; Aruffo and Seed 1987a, b; Seed and Aruffo, 1987), the system suffers from low transfection efficiencies and multiple labor-intensive screening and transfection rounds are needed (Munro and Maniatis, 1989). The development of the retroviral expression cloning method allowed for screening of a wide variety of cell lines and even primary cells, resulting in the discovery of a num-

Figure 2. Library construction and screening methods. mRNA from a cell line, an organ or a tissue is reverse-transcribed into cDNA and cloned in the pSinRep5 expression vector. The SinRep5 cDNA library is transfected into BHK cells together with a defective helper construct and viruses representing the cDNA library are released. BHK cells are infected with these viral particles resulting in the expression of one cDNA per cell. Cells are subsequently screened for proteins with a desired function. Membrane proteins can be screened by FACS analysis (left part). The one-gene-per-well format screens for secreted proteins (right part). Released recombinant viruses containing the desired RNA are amplified, and the recombinant RNA is isolated and sequenced. The virus can also be used for production of the selected protein.

ber of proteins (Kitamura et al., 1995; Whitehead et al., 1995). Major difficulties of the system are the relatively low expression levels, the decrease in representation of mRNAs of the library when cDNAs confer instability to the virus, or inhibit the reverse transcription event, and the time-consuming expansion of selected cells displaying a certain function. Moreover, cDNAs coding for toxic proteins will often get lost during this last step.

To circumvent the problems with existing library expression methods, a Sindbis-based screening system, referred to as DELphi, was developed (Koller et al., 2001). Alphaviral expression systems offer a number of advantages for functional expression cloning in mammalian cells. Using recombinant SIN virus for infection allows for high level and monoclonal expression of a cDNA because one virus is sufficient for a full replication cycle (Johnston et al., 1974). The virus can easily be amplified and the cDNA encoded recovered. The alphaviral-based functional cloning system is outlined in Figure 2. The total mRNA of a certain (diseased) tissue, organ, or cell line is converted to cDNA and cloned into the pSinRep5 SIN-based expression plasmid (Figure 1B) under the control of the viral subgenomic promoter (Breedendiek et al., 1993). In vitro transcribed RNA is electroporated into mammalian cells together with a defective helper construct (Figure 1B), leading to the generation of an alphavirus library. Every replication competent viral particle contains one RNA molecule encoding the structural proteins and one RNA encoding the viral replicase and a library derived cDNA. This viral library is used to infect a new layer of mammalian cells, which are subsequently screened for a desired function. Two screening methods have been developed resulting in the cloning of different genes (Figure 2). In the one gene per cell format, BHK cells are infected with the alphaviral library and incubated with a certain potential binding partner, such as a ligand or a specific antibody. Using fluorescence-activated cell sorting (FACS), labeled ligand- or antibody-binding virus infected cells may be isolated. Infective Sindbis virus particles harboring the corresponding cDNA that are released from the sorted cells can be amplified by infection of a new layer of BHK cells. The sequence of the specific ligand- or antibody binding receptor may then be obtained by RT-PCR of the RNA from the infected cells using primers binding to Sindbis sequences directly up- and downstream of the insert. Within one round of selection, viral clones encoding proteins recognized by monoclonal antibodies against

Figure 3. Cloning of CD16 cDNA from a complex cDNA library. cDNA from peripheral blood lymphocytes was cloned into the pSinRep5 expression vector and a Sindbis virus library was generated. BHK cells were infected and screened for CD16 with a labeled anti-CD16 antibody and dead cells were sorted out by propidium iodide (PI) staining. 18 cells out of 7.5×10^5 showed binding of anti-CD16 antibodies (A). FACS sorted cells were diluted on sub-confluent naïve BHK cell layers to amplify the virus resulting in over 20% CD16 positive cells after two virus passages (B, C). The recombinant viral RNA was isolated, reverse-transcribed and sequenced.

CD4, CD28, CD16 (Figure 3), and of unknown specificity could be isolated from a mouse spleen cell library. These results showed that genes could be cloned out of a complex library in only one screening step.

The 'one gene per well' screening method is used to clone new secreted proteins. The viral particles from the alphaviral library are used to infect a sub-confluent BHK layer in 96-well plates. Supernatant

containing secreted proteins encoded by the recombinant viruses are harvested and placed on a different indicator cell line. Their effect on differentiation, activation, cell death, enhanced survival, or migration of a specific cell type are subsequently assessed. The virus containing supernatants must be re-screened to clone the virus-encoded gene responsible for the observed activity. We screened e.g. for the activation and maturation of dendritic cells, important cells in immune responses, and found the tumor necrosis factor alpha, and an unknown protein, which is now further analyzed.

In conclusion, the features of the alphaviral expression system allowed us to develop a screening method for the rapid cloning of genes from complex libraries. Infectious viruses are readily obtained from infected cells and sufficient virus can be generated within 48 hours from a single infected cell to sequence the library-derived cDNA.

Vector development

The original and most obvious use of alphaviruses is the high-level production of recombinant proteins (Liljeström, 1991; Lundström, 1997). Although numerous Sindbis, Semliki Forest and Venezuelan equine encephalitis virus-based expression systems exist, novel sophisticated alphaviral-based expression vectors are being developed for specific applications. An interesting finding was the identification of mutations that render the viral replicase non-cytopathic. Via two different approaches, mutations in the nsp2 gene of the viral replicase have been identified rendering the replicase non-cytopathic for both SIN virus and SFV (Dryga et al., 1997; Agapov et al., 1998; Frolov et al., 1999; Perri et al., 2000). Weiss et al. (1980) established a persistently infected BHK cell culture by infecting cells with a virus preparation enriched in defective interfering particles (DI) (Strauss and Strauss, 1994). DIs are deleted forms of standard alphaviruses incapable of autonomous replication, and require co-infection of standard virus for their replication. Almost two decades later, Dryga et al. (1997) identified a single amino acid change in the nsp2 protein (Pro726Ser) that resulted in the persistent, non-cytopathic nature of the virus. Incorporation of this mutation in SIN expression vectors led to higher and prolonged marker protein expression than obtained with the wildtype replicase. Another strategy to select non-cytopathic SIN replicons was developed

by Agapov et al. (1998). A replicon encoding the puromycin N-acetyltransferase gene was transfected in BHK cells. Transfected cells were selected by puromycin. However, transfected cells could only survive when a mutation was introduced during replication that rendered the replicase non-cytopathic. This mutation was identified as a predicted proline to leucine amino-acid change in exactly the same locus as the mutation identified by Dryga et al. (1997). A similar strategy was applied to screen for new non-cytopathic mutations in SFV and Sindbis (Perri et al., 2000). Some of the identified mutations resulted in a decrease of viral RNA synthesis and packaging efficiency, but marker protein production was hardly changed in most of the mutants tested (Frolov et al., 1999; Perri et al., 2000). These non-cytopathic replicons with reduced effect on host cell metabolism have potential for long-term high-level expression in different fields of both research and biotechnology. Examples include gene therapy, neurobiology (Lundström, 1999, 2000) and viral research (Schlesinger, 2000).

We used the non-cytopathic mutation in the nsp2 gene, together with a temperature sensitive mutation in nsp4 (ts6) for the construction of a temperature-regulatable DNA-based alphaviral expression system (Boorsma et al., 2000). The double mutated replicon, referred to as pCytTS, makes the establishment of stable transfected cell lines possible that express recombinant proteins upon shift to temperatures lower than 35 °C, with maximal expression at 29 °C. No expression could be detected at 37 °C. The high stringency allowed the generation of stable cell lines expressing the strong pro-apoptotic protein RipDD upon induction. A variety of proteins have been produced in different cell lines (Table 1). Important advantages include the simple mode of induction for which no chemical inducer is required, the fast construction of stable cell lines because all elements for regulatable expression are present on one plasmid, and the high inducibility, even in transiently transfected cells. The precise regulation of expression by temperature allows the development of new bioprocess strategies for the production of 'difficult proteins', but also facilitates the study of toxic proteins. Lundström et al. (2001) introduced mutations in the non-structural proteins of SFV that rendered the virus less cytopathic and expression temperature sensitive. Infected BHK and primary neurons showed high and prolonged expression at 31 °C, but no expression could be detected at 37 °C. These mutant viruses will have applications in neurobiology, as described above, but may also res-

Table 1. Proteins expressed in different cell lines

Cell line		Expressed proteins	
name	origin	stable	transient
BHK21	Baby Hamster Kidney	GFP, SEAP, β IFN, EPO, AID56-GFP, RipDD, HIV gp140 Sindbis Structural Proteins	HIV gp160, SevC, CXCR3 LacZ
CHO-K1	Chinese Hamster Ovary	GFP	SEAP
293	Human Embryonic Kidney	GFP, EPO	
293T	Human Embryonic Kidney	GFP, EPO	
HepG2	Human Hepatoma		GFP, EPO
HT1080	Human Bone Marrow		GFP
	Human Foreskin Fibroblast		GFP, LacZ
COS-7	Green Monkey Kidney	GFP	SEAP, EPO
BF	Moue Embryo Fibroblast	GFP, SevC	
N2A	Mouse Neuroblastoma	GFP, AID56-GFP	
C2C12	Mouse Myoblast	GFP, EPO, AID56-GFP	SEAP
MC57	Mouse Fibroblast		GFP

GFP, green fluorescent protein; AID56-GFP, Amyloid- β Precursor Protein (APP) Intracellular Domain with N-terminal GFP fusion (Passer at al., 2000); EPO, erythropoietin; SEAP, secreted alkaline phosphatase; IFN, interferon, RipDD, death domain of the receptor interacting protein; HIV gp140, HIV glycoproteins lacking the membrane anchor; SevC, Sendai virus C-protein; LacZ, β -galactosidase.

ult in powerful SFV DNA-based inducible expression systems similar to the pCytTS vector.

Conclusions

Alphaviral vectors are used for a variety of applications. The ease of handling, its efficient RNA replication, and its broad host range make the alphaviral expression system the method of choice for high-level production of recombinant proteins. Although only a few papers describe the use of alphaviruses for large-scale protein production, the newest vectors with increased safety should make them an attractive alternative to conventional methods. Very promising results have been obtained using the vectors for vaccine development and gene therapy. We added two Sindbis-based technologies to the alphaviral toolbox. Our functional expression cloning system allows the rapid identification of cDNAs from complex libraries specifying a functional activity. Several features of the alphaviral expression system distinguishes this screening system from other methods, including high expression, super-infection exclusion, and strong amplification of selected recombinant viruses. The temperature regulatable expression system combines the high expression levels of alphaviral vectors with the construction of stable cell lines for the production and study of pro-apoptotic proteins. The latest

developments in alphaviral expression systems result in useful tools for the present era of gene discovery, gene therapy, vaccine development and recombinant protein production.

Acknowledgement

We thank Professor Sondra Schlesinger, Washington University (St. Louis, MO) for helpful discussions.

References

- Agapov EV, Frolov I, Lindenbach BD, Pragai BM, Schlesinger S & Rice CM (1998) Non-cytopathic Sindbis virus RNA vectors for heterologues gene expression. *Proc Nat Acad Sci USA* 95: 12989–12994.
- Altman-Hamamdziec S, Groseclose C, Ma JX, Hamamdziec D, Vridavanam NS, Middaugh LD, Parratto NP & Sallee FR (1997) Expression of beta-galactosidase in mouse brain: utilization of a novel nonreplicative Sindbis virus vector as a neuronal gene delivery system. *Gene Ther* 4: 815–822.
- Aruffo A & Seed B (1987a) Molecular cloning of two CD7 (T-cell leukemia antigen) cDNAs by a COS cell expression system. *EMBO J* 6: 3313–3316.
- Aruffo A & Seed B (1987b) Molecular cloning of a CD28 cDNA by a high-efficiency COS cell expression system. *Proc Natl Acad Sci USA* 84: 8573–8577.
- Asselin-Paturel C, Lassau N, Guinebretiere JM, Zhang J, Gay F, Bex F, Hallez S, Leclerc J, Peronneau P, Mami-Chouaib F & Chouaib S (1999) Transfer of the murine interleukin-12 gene in vivo

- by a Semliki Forest virus vector induces B16 tumor regression through inhibition of tumor blood vessel formation monitored by Doppler ultrasonography. *Gene Ther* 6: 606–615.
- Berglund P, Quesada-Rolander M, Putkonen P, Biberfeld G, Thorstensson R & Liljeström P (1997) Outcome of immunization of cynomolgus monkeys with recombinant Semliki Forest virus encoding human immunodeficiency virus type 1 envelope protein and challenge with a high dose of SHIV-4 virus. *AIDS Res Hum Retroviruses* 13: 1487–1495.
- Boorsma M, Nieba L, Koller D, Bachmann MF, Bailey JE & Renner WA (2000) A temperature-regulated replicon-based DNA expression system. *Nat Biotechnol* 18: 429–432.
- Breidenbeek PJ, Frolov I, Rice CM & Schlesinger S (1993) Sindbis virus expression vectors: Packaging of RNA replicons by using defective helper RNAs. *J Virol* 67: 6439–6446.
- Colmenero P, Liljeström P & Jondal M (1999) Induction of P815 tumor immunity by recombinant Semliki Forest virus expressing the P1A gene. *Gene Ther* 6: 1728–1733.
- Daemen T, Pries F, Bungener L, Kraak M, Regts J & Wilschut J (2000) Genetic immunization against cervical carcinoma: induction of cytotoxic T lymphocytes activity with a recombinant alphavirus vector expressing human papillomavirus type 16 E6 and E7. *Gene Ther* 7: 1859–1866.
- Datwyler DA, Eppenberger HM, Koller D, Bailey JE & Magyar JP (1999) Efficient gene delivery into adult cardiomyocytes by recombinant Sindbis virus. *J Mol Med* 77: 859–864.
- Davis NL, Willis LV, Smith JF & Johnston RE (1989) In vitro synthesis of infectious Venezuelan equine encephalitis virus RNA from a cDNA clone: analysis of a viable deletion mutant. *Virology* 171: 189–204.
- Davis NL, Caley JJ, Brown KW, Betts MR, Irlbeck DM, McGrath KM, Connell MJ, Montefiori DC, Frelinger JA, Swanson R, Johnson PR & Johnston RE (2000) Vaccination of macaques against pathogenic simian immunodeficiency virus with Venezuelan equine encephalitis virus replicon particles. *J Virol* 74: 371–378.
- Dryga SA, Dryga OA & Schlesinger S (1997) Identification of mutations in a Sindbis virus variant able to establish persistent infection in BHK cells: the importance of a mutation in the nsP2 gene. *Virology* 228: 74–83.
- Ehrengruber MU, Lundström K, Schweitzer C, Heuss C, Schlesinger S & Gähwiler BH (1999) Recombinant Semliki Forest virus and Sindbis virus efficiently infect neurons in hippocampal slice cultures. *Proc Natl Acad Sci USA* 96: 7041–7046.
- Fleeton MN, Chen M, Berglund P, Rhodes G, Parker SE, Murphy M, Atkins GJ & Liljeström P (2001) Self-replicative RNA vaccines elicit protection against Influenza A virus, respiratory syncytial virus, and a Tickborne encephalitis virus. *J Infect Dis* 183: 1395–1398.
- Frolov I, Hoffman TA, Pragai BM, Dryga SA, Huang HV, Schlesinger S & Rice CM (1996) Alphavirus-based expression vectors: strategies and applications. *Proc Natl Acad Sci USA* 93: 11371–11377.
- Frolov I, Agapov E, Hoffman TA, Pragai BM, Lippa M, Schlesinger S & Rice CM (1999) Selection of RNA replicons capable of persistent noncytopathic replication in mammalian cells. *J Virol* 73: 3854–3865.
- Gardner JP, Frolov I, Perri S, Ji Y, MacKichan ML, zur Megede J, Chen M, Belli BA, Driver DA, Sherrill S, Greer CE, Otten GR, Barnett SW, Liu MA, Dubensky TW & Polo JM (2000) Infection of human dendritic cells by a Sindbis virus replicon vector is determined by a single amino acid substitution in the E2 glycoprotein. *J Virol* 74: 11849–11857.
- Garoff H & Li KJ (1998) Recent advances in gene expression using alphavirus vectors. *Curr Opin Biotechnol* 9: 464–469.
- Hahn CS, Hahn YS, Braciale TJ & Rice CM (1992) Infectious Sindbis virus transient expression vectors for studying antigen processing and presentation. *Proc Natl Acad Sci USA* 89: 2679–2783.
- Hardy PA, Mazzini MJ, Schweitzer C, Lundström K & Glode LM (2000) Recombinant Semliki forest virus infects and kills human prostate cancer cell lines and prostatic duct epithelial cells *ex vivo*. *Int J Mol Med* 5: 241–245.
- Herweijer H, Latendress JS, Williams P, Zhang G, Danko I, Schlesinger S & Wolff JA (1995) A plasmid-based self-amplifying Sindbis virus vector. *Hum Gene Ther* 6: 1161–1167.
- Johnston RE, Wan K & Bose HR (1974) Homologous interference induced by Sindbis virus. *J Virol* 14: 1076–82.
- Kaariainen L & Ranki M (1984) Inhibition of cell functions by RNA-virus infections. *Annu Rev Microbiol* 38: 91–109.
- Kay MA, Glorioso J & Naldini L (2001) Viral vectors for gene therapy: the art of turning infectious agents into vehicles of therapeutics. *Nat Med* 7: 33–40.
- Kitamura T, Onishi M, Kinoshita S, Shibuya A, Miyajima A & Nolan GP (1995) Efficient screening of retroviral cDNA expression libraries. *Proc Natl Acad Sci USA* 92: 9146–9150.
- Klimp AH, van de Vaart E, Lansink PO, Withoff S, de Vries EGE, Scherphof GL, Wildschut J & Daemen T (2001) Activation of peritoneal cells upon *in vivo* transfection with a recombinant alphavirus expressing GM-CSF. *Gene Ther* 8: 300–307.
- Koller et al. (2001) A high-throughput alphavirus-based expression cloning system for mammalian cells. *Nature Biotechnology* 19: 851.
- Leitner WW, Ying H, Driver DA, Dubensky TW & Restifo NP (2000) Enhancement of tumor-specific immune response with plasmid DNA replicon vectors. *Cancer Res* 60: 51–55.
- Lijima Y, Ohno K, Ikeda H, Sawai K, Levin B & Meruelo D (1999) Cell-specific targeting of a thymidine kinase/ganciclovir gene therapy system using a recombinant Sindbis virus vector. *Int J Cancer* 80: 110–118.
- Liljeström P & Garoff H (1991) A new generation of animal cell expression vectors based on the Semliki Forest virus replicon. *Bio/Technology* 9: 1356–1361.
- Liljeström P, Lusa S, Huylebroeck D & Garoff H (1991) *In vitro* mutagenesis of a full-length cDNA clone of Semliki Forest virus: the small 6,000-molecular-weight membrane protein modulates virus release. *J Virol* 65: 4107–4113.
- Lundström K (1997) Alphaviruses as expression vectors. *Curr Opin Biotechnol* 8: 578–582.
- Lundström K, Richards JG, Pink JR & Jenck F (1999) Efficient *in vivo* expression of a reporter gene in rat brain after injection of recombinant replication-deficient Semliki Forest virus. *Gene Ther* 6: 15–23.
- Lundström K, Rotmann D, Hermann D, Schneider EM & Ehrengruber MU (2001) Novel mutant Semliki Forest virus vectors: gene expression and localization studies in neuronal cells. *Histochem Cell Biol* 115: 83–91.
- Lundström K (1999) Alphaviruses as tools in neurobiology and gene therapy. *J Recept Signal Transduct Res* 19: 673–686.
- Lundström K (2000) Semliki Forest virus vectors: Versatile applications in neurobiology. *Res Adv In Neurochem* 1: 1–11.
- MacDonald GH & Johnston RE (2000) Role of dendritic cell targeting in Venezuelan equine encephalitis virus pathogenesis. *J Virol* 74: 914–922.
- Maletic-Savatic M, Malinow R & Svoboda K (1999) Rapid dendritic morphogenesis in CA1 hippocampal dendrites induced by synaptic activity. *Science* 283: 1923–1927.

- Mossman SP, Bex F, Berglund P, Arthos J, O'Neil SP, Riley D, Maul DH, Bruck C, Momin P, Burny A, Fultz PN, Mullins JJ, Liljeström P & Hoover EA (1996) Protection against lethal simian immunodeficiency virus SIVsmmPBj14 disease by a recombinant Semliki Forest virus gp160 vaccine and by a gp120 subunit vaccine. *J Virol* 70: 1953–1960.
- Munro S & Maniatis T (1989) Expression cloning of the murine interferon gamma receptor cDNA. *Proc Natl Acad Sci USA* 86: 9248–52.
- Murphy AM, Morris-Downes MM, Sheahan BJ & Atkins GJ (2000) Inhibition of human lung carcinoma cell growth by apoptosis induction using Semliki Forest virus recombinant particles. *Gene Ther* 7: 1477–1482.
- Ohno K, Sawai K, Lijima Y, Levin B & Meruelo D (1997) Cell-specific targeting of Sindbis virus vectors displaying IgG-binding domains of protein A. *Nat Biotechnol* 15: 763–767.
- Okayama H & Berg P (1983) A cDNA cloning vector that permits expression of cDNA inserts in mammalian cells. *Mol Cell Biol* 3: 280–289.
- Passer B, Pellegrini L, Russo C, Siegel RM, Lenardo MJ, Schettini G, Bachmann MF, Tabaton M & D'Adamio L (2000) Generation of an apoptotic intracellular peptide by γ -secretase cleavage of Alzheimer's β -amyloid precursor protein. *J Alzheimer's Dis* 2 (in press).
- Perri S, Driver DA, Gardner JP, Sherrill S, Belli BA, Dubensky TW & Polo JM (2000) Replicon vectors derived from Sindbis virus and Semliki Forest virus that establish persistent replication in host cells. *J Virol* 74: 9802–9807.
- Polo JM, Belli BA, Driver DA, Frolov I, Sherrill S, Hariharan MJ, Townsend K, Perri S, Mento SJ, Jolly DF, Chang SMW, Schlesinger S & Dubensky TW (1999) Stable alphavirus packaging cell lines for Sindbis virus- and Semliki Forest virus-derived vectors. *Proc Natl Acad Sci USA* 96: 4598–4603.
- Raju R & Huang HV (1991) Analysis of Sindbis virus promoter recognition in vivo, using novel vectors with two subgenomic mRNA promoters. *J Virol* 65: 2501–25100.
- Rice CM, Levis R, Strauss JH & Huang HV (1987) Production of infectious RNA transcripts from Sindbis virus cDNA clones: Mapping of lethal mutations, rescue of a temperature-sensitive marker, and in vitro mutagenesis to generate defined mutants. *J Virol* 61: 3809–3819.
- Seed B & Aruffo A (1987) Molecular cloning of the CD2 antigen, the T-cell erythrocyte receptor, by a rapid immunoselection procedure. *Proc Natl Acad Sci USA* 84: 3365–3369.
- Simonsen H & Lodish HF (1994) Cloning by function: expression cloning in mammalian cells. *Trends Pharmacol Sci* 15: 437–441.
- Schlesinger S (2000) Alphavirus Expression Vectors. In: Maramorosch K, Murphy FA & Shatkin AJ (eds.) *Advances in Virology*, Vol. 55 (pp. 565–577).
- Schlesinger S & Dubensky TW (1999) Alphavirus vectors for gene expression and vaccines. *Curr Opin Biotech* 10: 434–439.
- Schlesinger S & Schlesinger MJ (1996) Togaviridae: the viruses and their replication. In: Knipe DM & Howley PM (eds.) *Fields Virology*, Vol. I (pp. 825–841) Lippincott-Raven, Philadelphia.
- Strauss JH & Strauss EG (1994) The alphaviruses: gene expression, replication and evolution. *Microbiol Rev* 58: 491–562.
- Vassar R, Bennet BD, Babu-Khan S, Kahn S, Mendiaz EA, Denis P, Teplow DB, Ross S, Amarante P, Loeloff R, Luo Y, Fisher S, Fuller J, Edenson S, Lile J, Jarosinski MA, Biere AL, Curran E, Burgess T, Louis JC, Collins F, Treanor J, Rogers G & Citron M (1999) Beta-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. *Science* 286: 735–741.
- Whitehead I, Kirk H & Kay R (1995) Expression cloning of oncogenes by retroviral transfer of cDNA libraries. *Mol Cell Biol* 15: 704–710.
- Wong GG, Witek JS, Temple PA, Wilkens KM, Leary AC, Luxenberg DP, Jones SS, Brown EL, Kay RM, Orr EC et al. (1985) Human GM-CSF: molecular cloning of the complementary DNA and purification of the natural and recombinant proteins. *Science* 228: 810–815.
- Xiong C, Levis R, Shen P, Schlesinger S, Rice CM & Huang HV (1989) Sindbis virus: an efficient, broad host range vector for gene expression in animal cells. *Science* 243: 1188–1191.
- Ying H, Zaks TZ, Wang RF, Irvine KR, Kammula US, Marincola FM, Leitner WW & Restifo NP (1999) Cancer therapy using a self-replicating RNA vaccine. *Nat Med* 5: 823–827.